

# The role of Rubisco and cell walls in the interspecific variation in photosynthetic capacity

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**Abstract** Photosynthetic capacity is known to vary considerably among species. Its physiological cause and ecological significance have been one of the most fundamental questions in plant ecophysiology. We studied the contents of Rubisco (a key enzyme of photosynthesis) and cell walls in leaves of 26 species with a large variation in photosynthetic rates. We focused on photosynthetic nitrogen-use efficiency (PNUE, photosynthetic rate per nitrogen), which can be expressed as the product of Rubisco-use efficiency (RBUE, photosynthetic rate per Rubisco) and Rubisco nitrogen fraction (RNF, Rubisco nitrogen per total leaf nitrogen). RBUE accounted for 70% of the interspecific variation in PNUE. The variation in RBUE was ascribed partly to stomatal conductance, and other factors such as mesophyll conductance and Rubisco kinetics might also be involved. RNF was also significantly related to PNUE but the correlation was relatively weak. Cell wall nitrogen fraction (WNF, cell wall nitrogen per total leaf nitrogen) increased with increasing leaf mass per area, but there was no correlation between RNF and WNF. These results suggest that nitrogen allocation to cell walls does not explain the variation in PNUE. The difference in PNUE was not caused by a sole factor that was markedly different among

species but by several factors each of which was slightly disadvantageous in low PNUE species.

**Keywords** Cell wall nitrogen · Leaf trait convergence · Life form · Nitrogen partitioning · Photosynthetic nitrogen-use efficiency

## Abbreviations

$C_i/C_a$	Ratio of CO <sub>2</sub> concentration in intercellular spaces to that in air
LLS	Leaf life span
LMA	Leaf mass per area
$N$	Leaf nitrogen
$R$	Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase)
RNF	Rubisco nitrogen fraction
$P$	Photosynthetic rate
PNUE	Photosynthetic nitrogen-use efficiency
RBUE	Rubisco-use efficiency
WM	Cell wall mass
WN	Cell wall nitrogen
WNF	Cell wall nitrogen fraction
Subscripted “area”	Per unit leaf area
Subscripted “mass”	Per unit mass

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## Introduction

It is well known that there is a large variation in photosynthetic capacity across plants. A recent global survey dealing with 1% of vascular plant species on Earth revealed that photosynthetic capacity varied by 120- and 40-fold when expressed on a dry mass and a leaf area basis, respectively (Glopanet; Wright et al. 2004). Within-species variation in

photosynthetic capacity has been ascribed to leaf nitrogen concentration (Field and Mooney 1986; Evans 1989); as about one-half of leaf nitrogen is involved in the photosynthetic apparatus; there is a strong correlation between photosynthetic capacity and leaf nitrogen concentration within species. However, there is a considerable interspecific variation in photosynthetic capacity even when compared at the same nitrogen concentration. Photosynthetic nitrogen-use efficiency (PNUE, photosynthetic rate per unit leaf nitrogen) has thus attracted much attention as we aim to improve our understanding of the inherent variation in photosynthetic capacity among species (Field and Mooney 1986; Pons et al. 1994; Hikosaka 2004). In the Glopnet survey, PNUE varied by 40-fold (Wright et al. 2004) and was closely linked with other leaf traits (Wright et al. 2005). For example, PNUE was negatively correlated with leaf mass per area and leaf life span (Hikosaka 2004).

Studies on the physiological cause for the variation in PNUE have focused on Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) because it catalyzes the limiting step for photosynthetic capacity (Farquhar et al. 1980) and accounts for a large fraction of leaf nitrogen (Evans and Seemann 1989). PNUE is higher if (1) more leaf nitrogen is allocated to Rubisco, or (2) Rubisco is used more efficiently for carbon fixation (Hikosaka and Hirose 2000). Rubisco-use efficiency (RBUE), defined as photosynthetic rate per unit Rubisco, is higher if the leaf has a higher concentration of the substrate,  $\text{CO}_2$ , at the carboxylation site or better kinetic properties of Rubisco (Field and Mooney 1986; Evans 1989; Hikosaka 2004).

In the past decade, many authors have analyzed the mechanisms underlying interspecific variation in PNUE. Most such studies have demonstrated that less leaf nitrogen is allocated to Rubisco in low-PNUE species (Hikosaka et al. 1998; Poorter and Evans 1998; Westbeek et al. 1999; Pons and Westbeek 2004; Warren and Adams 2004b; Warren et al. 2006; Feng et al. 2007; Feng 2008). For example, the Rubisco nitrogen fraction in leaves (RNF, Rubisco nitrogen per unit leaf nitrogen) was found to be 23% higher in an annual herb than in an evergreen tree (Hikosaka et al. 1998). Recently, two papers proposed a trade-off in nitrogen partitioning between photosynthesis and cell walls. They reported negative relationships between RNF and cell wall nitrogen fraction (WNF) across leaves of a perennial herb (Onoda et al. 2004) and across evergreen and deciduous *Quercus* species (Takashima et al. 2004). This trade-off suggests that leaves with longer life spans need more investment of nitrogen in cell walls to become tougher, at the sacrifice of photosynthetic capacity (Reich et al. 1991; Hikosaka 2005). It has also been indicated that mesophyll conductance for  $\text{CO}_2$  diffusion is responsible for the variation in PNUE (Lloyd et al. 1992; Hikosaka et al. 1998; Warren and Adams 2006; Terashima et al. 2006; Flexas

et al. 2008). Warren (2008) showed that the drawdown of the  $\text{CO}_2$  concentration from intercellular spaces to the carboxylation site was greater in sclerophytes than in non-sclerophytes. Lower mesophyll conductance may reduce RBUE and thus PNUE.

Although a number of studies on the mechanisms of variation in PNUE have been conducted, each dealt with only a limited number of species (ten or fewer) and the variation in PNUE was small (fourfold or smaller). Therefore, the quantitative contribution of each factor to the total variation in PNUE remains unclear. The aim of the present study was to find a general pattern in the variation of factors related to PNUE. We studied 26 temperate species from annual herbs to evergreen conifers with a large variation in leaf traits including PNUE. Determining gas exchange characteristics and concentrations of leaf nitrogen and Rubisco, we assessed the contribution of RBUE and RNF to the interspecific variation in PNUE. Furthermore, we tested whether the trade-off in nitrogen partitioning between photosynthesis and cell walls held across the large variation in PNUE.

## Materials and methods

We studied 26 plant species growing in Aobayama-hill, Aoba, Japan ( $38^\circ 15' \text{N}$ ,  $140^\circ 51' \text{E}$ , 50–150 m above sea level). Mean annual temperature and precipitation in 2001–2007 were  $12.5^\circ \text{C}$  and 1,228.6 mm, respectively. The 26 species included two annual herbs, six perennial herbs, seven deciduous trees, two deciduous shrubs, four evergreen broad-leaved trees, three evergreen broad-leaved shrubs and two evergreen conifers (Table S1). The plants grew in various habitats: forest gaps, forest edges, disturbed area such as roadside, and an old field in our botanical gardens. We selected sun leaves that had been exposed to direct sunlight at least for several hours per day. We used three to six fully expanded young leaves per species for photosynthetic measurements (four leaves in most cases). We tried to select each leaf from different individuals, but in some species leaves from one individual were used. We ignored between-individual differences.

Photosynthetic rates were determined between 0800 and 1500 hours on 5 sunny days from 13 August to 2 September 2003 with a portable photosynthesis measurement system (Li-6400, LiCor, Lincoln, NE) with an artificial light source. Steady-state measurements were made following equilibrium (longer than 4 min) under a photosynthetic photon flux density of  $1,000\text{--}2,000 \mu\text{mol m}^{-2} \text{s}^{-1}$  (we used lower light intensities for species with low photosynthetic rates to avoid photoinhibition), leaf temperature of  $25.9 \pm 1.0^\circ \text{C}$  (mean and standard deviation),  $\text{CO}_2$  concentration in the chamber ( $C_a$ ) of  $372.0 \pm 5.2 \mu\text{mol mol}^{-1}$ , and a vapor pressure deficit of  $1.27 \pm 0.30 \text{ kPa}$ .

After photosynthetic measurements, the leaves were collected and brought to the laboratory. Leaf discs with 1 cm diameter were punched out. Three discs were dried in an oven at 70°C for more than 3 days. Others were frozen in liquid nitrogen and stored in a freezer at –80°C. Leaf dry mass and nitrogen concentrations were determined for the dried samples with an NC analyzer (NC-80, Shimadzu, Kyoto). For species with small leaves (*Ilex*, *Abies* and *Cryptomeria*), leaves were scanned and their area was measured. As we could not obtain leaf discs from these species, the whole leaves that were used in photosynthetic measurements were dried and subjected to dry mass and nitrogen measurements. For Rubisco and cell wall analyses, we sampled adjacent leaves, which were stored in the freezer after leaf area measurements.

Rubisco concentration, cell wall mass, and cell wall nitrogen concentration were determined according to Takashima et al. (2004) with modifications. Frozen leaf discs (one disc in most cases) or a leaf were powdered in liquid nitrogen in a mortar with a pestle, and homogenized in 1 mL 100 mM Bicine buffer (pH 8.0) with 5 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 g for 30 min and the supernatant was applied to SDS polyacrylamide gel electrophoresis (SDS-PAGE) for determination of Rubisco content (see Hikosaka et al. 1998 for details). The pellet was washed with the same buffer again and then washed with water to remove SDS. As this procedure could not completely remove chlorophylls in some cases, we used PAW (phenol:acetic acid:water = 2:1:1, w:v:v; Fry 1988) for all leaves. The pellet was treated with PAW three times (1 h, overnight, and 1 h, respectively). PAW is a strong solvent and we considered that nitrogenous compounds other than cell wall proteins were successfully removed. The pellet was then washed with ethanol until there was no smell of phenol. The dry mass and nitrogen content determined with an NC analyzer (Vario EL-III, Elementar, Germany) were regarded as the mass and nitrogen of cell walls, respectively. Values of cell wall nitrogen obtained using this procedure were somewhat lower than those obtained by the method of Takashima et al. (2004), suggesting that our method might underestimate cell wall nitrogen concentrations (see Takashima et al. 2004 for discussion of estimation of cell wall nitrogen). On the other hand, as we did not remove starch from the pellet, cell wall mass in the present study might be overestimated, though the proportion of starch was considered to be small. This procedure could not extract Rubisco from leaves of three species (as judged by SDS-PAGE). In these cases cell wall nitrogen might be overestimated because some soluble proteins might have been precipitated with phenolic compounds, and we did not use values of cell wall parameters for these species.

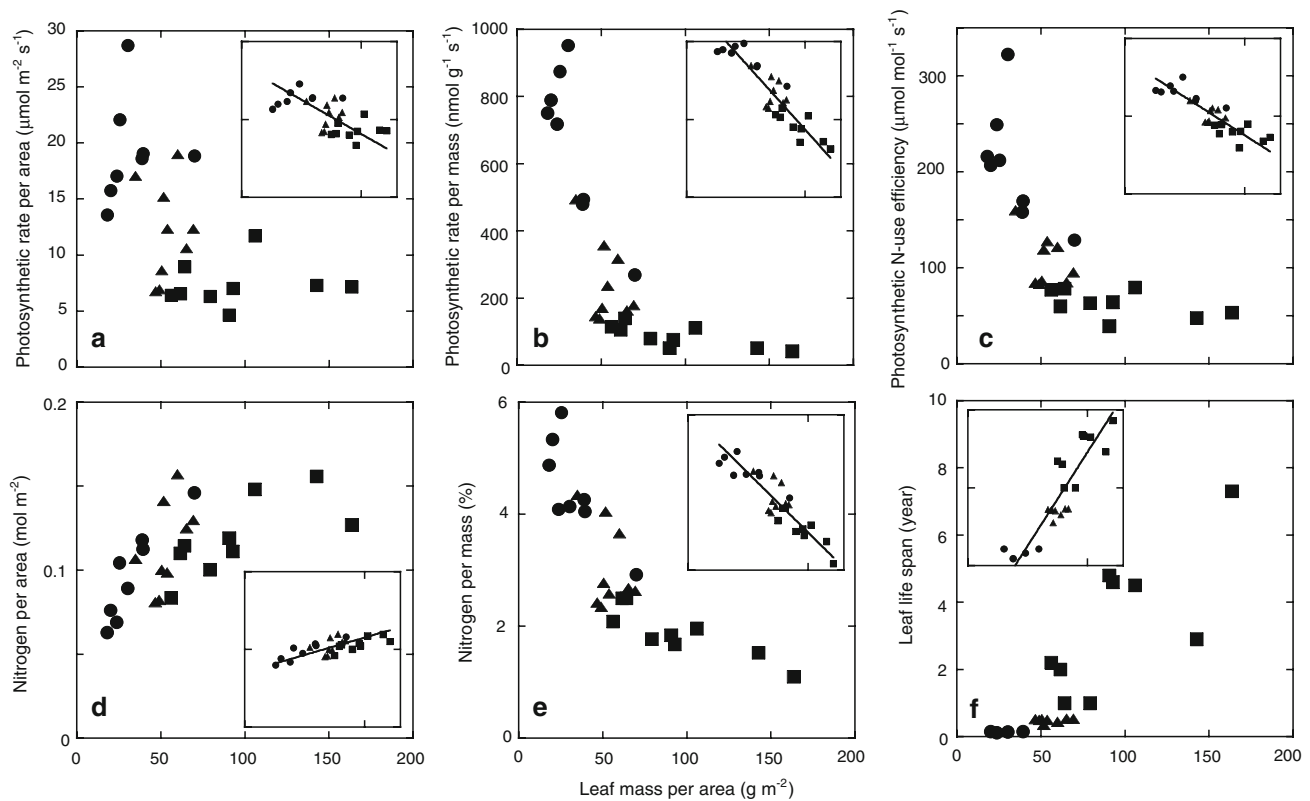
Leaf life span was measured in 2003–2007. For herbaceous species and *Morus australis*, five to ten newly emerged leaves were marked with colored wires and their presence was checked every 1 or 2 weeks in 2003 or 2006. Leaf life span was defined as the time taken for one-half of the leaves to be lost. For deciduous trees except for *M. australis*, the duration between spring flush and autumn fall in 2007 was regarded as leaf life span. For evergreen species, leaf life span was estimated using leaves and scars in 2006 with the aid of colored wires on branches marked in 2003. We could not determine leaf life span of some species because of mowing or herbivory.

We used real and  $\log_{10}$ -transformed values for regression analyses. Standardized major axis regression analyses (Warton et al. 2006) were performed using SMATR (Falster et al. 2006). Regression analyses were also performed for phylogeny-independent contrasts (PIC; Felsenstein 1985) using COMPARE (Martins 2004). Phylogenetic trees were constructed according to Stevens (2008) for the family level, Lamb Frye and Kron (2003) for Polygonaceae, Hasebe et al. (1998) for Aceraceae, and Ohyama et al. (1999) for Fagaceae species. Evolutionary rate was ignored in the analyses. As there were two uncertain points in the phylogeny (Fig. S1), we performed calculations for nine possible phylogenetic trees and calculated the mean of the correlation coefficients.

The effect of stomatal conductance on Rubisco-use efficiency was calculated using the biochemical model of the Rubisco-limited photosynthetic rate (Farquhar et al. 1980), with values of tobacco Rubisco kinetics that were estimated assuming mesophyll conductance being infinite (von Caemmerer et al. 1994). Chloroplast CO<sub>2</sub> concentration was also estimated as a function of photosynthetic rate per unit Rubisco with values of tobacco Rubisco kinetics that were estimated using actual mesophyll conductance (von Caemmerer et al. 1994).

## Results

There was a large variation in leaf traits among species (Fig. 1; Table S1). Photosynthetic rate per leaf area ( $P_{\text{area}}$ ) and PNUE varied from 4.7 to 28.7  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 39.3 to 322.4  $\mu\text{mol mol}^{-1} \text{s}^{-1}$ , respectively. The variation in PNUE in the present study was greater than that in earlier studies that analyzed its biochemical causes (e.g., twofold in Poorter and Evans 1998; fourfold in Pons and Westbeek 2004). Herbaceous species tended to have higher photosynthetic rates than evergreen woody species, and deciduous woody species were intermediate. Most leaf traits were closely related with each other across species (Table S2). These trends were consistent with those observed in earlier studies (e.g., Wright et al. 2004; Reich et al. 1992, 1997).



**Fig. 1** Relationships between leaf traits. Photosynthetic rate per area (**a**;  $P_{\text{area}}$ ), per mass (**b**;  $P_{\text{mass}}$ ), per nitrogen (**c**; PNUE, photosynthetic nitrogen-use efficiency), leaf nitrogen per area (**d**;  $N_{\text{area}}$ ), leaf nitrogen per mass (**e**;  $N_{\text{mass}}$ ) and leaf life span (**f**; LLS) are shown as a function of leaf mass per area (LMA). Circles Herbs, triangles deciduous woody species, squares evergreen woody species. Insets Same variables both on  $\log_{10}$ -transformed scale; full scale of LMA is

10–200  $\text{g m}^{-2}$  and others are scaled where a graduation means  $10^0$ . Regression lines were obtained with standardized major axis regression ( $\log Y = 2.57 - 0.877 \log X$ ,  $r^2 = 0.36$  for  $P_{\text{area}}$ -LMA;  $\log Y = 5.26 - 1.70 \log X$ ,  $r^2 = 0.82$  for  $P_{\text{mass}}$ -LMA;  $\log Y = 3.72 - 0.98 \log X$ ,  $r^2 = 0.74$  for PNUE-LMA;  $\log Y = -1.74 + 0.446 \log X$ ,  $r^2 = 0.50$  for  $N_{\text{area}}$ -LMA;  $\log Y = 1.77 - 0.761 \log X$ ,  $r^2 = 0.82$  for  $N_{\text{mass}}$ -LMA;  $\log Y = -4.36 + 2.41 \log X$ ,  $r^2 = 0.78$  for LLS-LMA)

In brief, species with higher leaf mass per area (LMA) had lower  $P_{\text{area}}$ , photosynthetic rates per mass ( $P_{\text{mass}}$ ), PNUE and leaf nitrogen per mass ( $N_{\text{mass}}$ ), higher leaf nitrogen per area ( $N_{\text{area}}$ ) and leaf life span (LLS) (Fig. 1). In most cases, correlations did not differ among life forms. Correlation coefficients tended to be stronger when the values were log-transformed (Table S2). A correlation analysis for PIC (Felsenstein 1985) showed tendencies similar to those of the usual analyses (Table S2), suggesting that our results were independent of phylogenetic constraints.

Rubisco per leaf area ( $R_{\text{area}}$ ) varied from 0.97 to  $3.57 \text{ g m}^{-2}$ .  $R_{\text{area}}$  was not different among life forms (Fig. 2a; Table S1). Rubisco nitrogen fraction (RNF, fraction of leaf nitrogen in Rubisco), which varied from 13.2 to 33.1%, was higher in some herbaceous species but similar among others (Fig. 2b; Table S1). Both parameters were either weakly or not significantly correlated with other leaf traits (Table S2); for example, neither were significantly correlated with LMA (Fig. 2a, b).

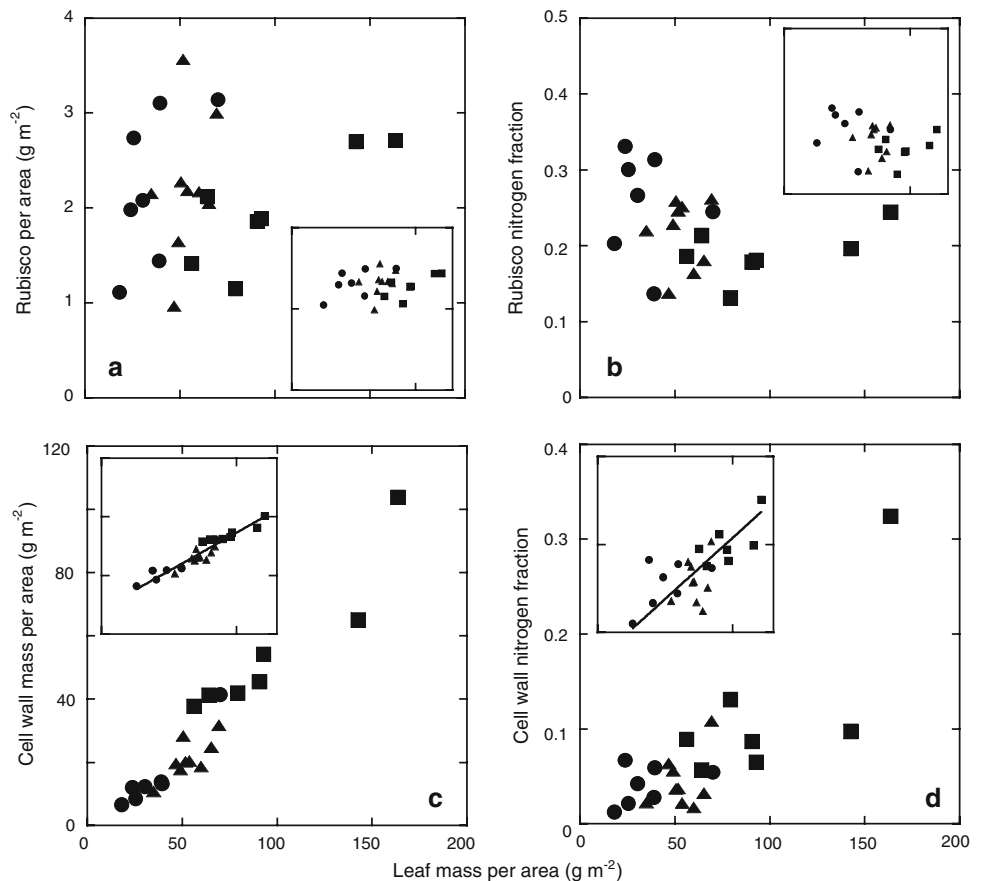
Cell wall mass per area (WM) and cell wall nitrogen fraction (WNF) varied by  $6.6\text{--}104 \text{ g m}^{-2}$  and  $1.3\text{--}32.4\%$ , respectively (Fig. 2c,d; Table S1). Evergreen species

tended to invest more biomass and nitrogen in cell walls. The slope of the log-transformed relationship between WM and LMA was greater than 1 (1.3), indicating that the fraction of leaf mass in cell walls increased with increasing LMA (Fig. 2c; Table S2). WNF also increased with increasing LMA (Fig. 2d). The nitrogen concentration of cell walls varied from 0.15 to 0.70% (Table S1) and was not significantly correlated with leaf traits other than parameters of cell wall nitrogen (Table S2).

PNUE was analyzed as the product of RBUE and RNF. PNUE was almost proportional to RBUE, which accounted for 70% of the variation in PNUE (Fig. 3a). PNUE was also significantly correlated with RNF, but the correlation was relatively weak ( $r^2 = 0.27$ ; Fig. 3b). Within each life form, correlation between PNUE and RNF was very weak.

Cell wall parameters were well correlated with PNUE (Table S2). WNF was negatively correlated with PNUE (Fig. 3c). However, there was no significant correlation between WNF and RNF (Fig. 3d). These results suggest that nitrogen allocation to cell wall does not sacrifice nitrogen allocation to photosynthesis, and thus cell wall nitrogen itself may not directly affect PNUE.

**Fig. 2** Rubisco per area (**a**;  $R_{\text{area}}$ ), Rubisco nitrogen fraction (**b**; RNF, leaf nitrogen allocated to Rubisco), Cell wall mass per area (**c**; WM) and cell wall nitrogen fraction (**d**; WNF) as a function of leaf mass per area (LMA). *Circles* Herbs, *triangles* deciduous woody species, *squares* evergreen woody species. *Insets* Same variables both on  $\log_{10}$ -transformed scale; full scale of LMA is 10–200  $\text{g m}^{-2}$  and others are scaled where a graduation means  $10^n$ . *Regression lines* were obtained with standardized major axis regression ( $\log Y = -0.876 + 1.302 \log X$ ,  $r^2 = 0.91$  for WM–LMA;  $\log Y = -3.708 + 1.392 \log X$ ,  $r^2 = 0.48$  for WNF–LMA)



## Discussion

There was a large variation in leaf traits in the present study; ranges of the  $\log_{10}$ -transformed values in LMA, LLS,  $P_{\text{area}}$ ,  $P_{\text{mass}}$  and PNUE were 0.96, 1.77, 0.79, 1.35 and 0.91, respectively (Fig. 1; Table S1), where the range of 1 means that the non-transformed values of the trait varied by one order of magnitude. Variations in these leaf traits cover almost half of the variations observed in the global survey (Glopanet; Wright et al. 2004); the ranges were 2.03, 2.51, 1.60, 2.08 and 1.60 for LMA, LLS,  $P_{\text{area}}$ ,  $P_{\text{mass}}$  and PNUE, respectively. Furthermore, correlations among leaf traits in the present study were very similar to the Glopanet survey. Our data set thus represents typical leaf trait variation.

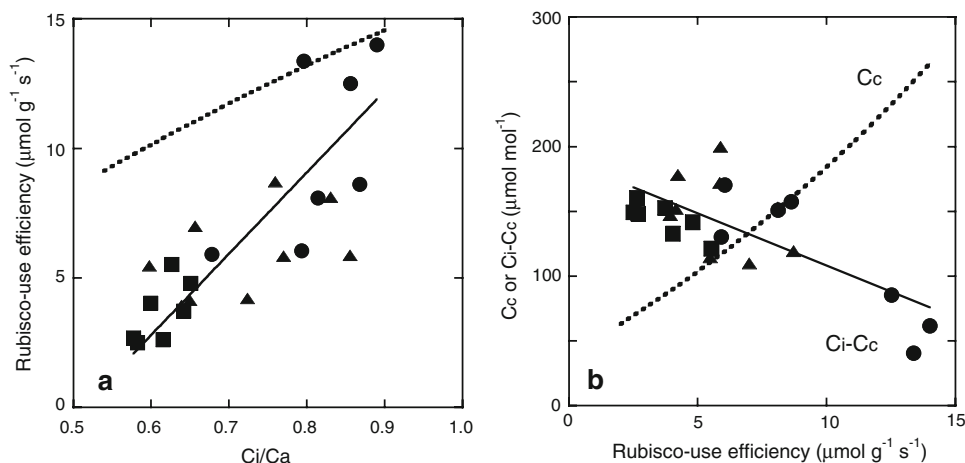
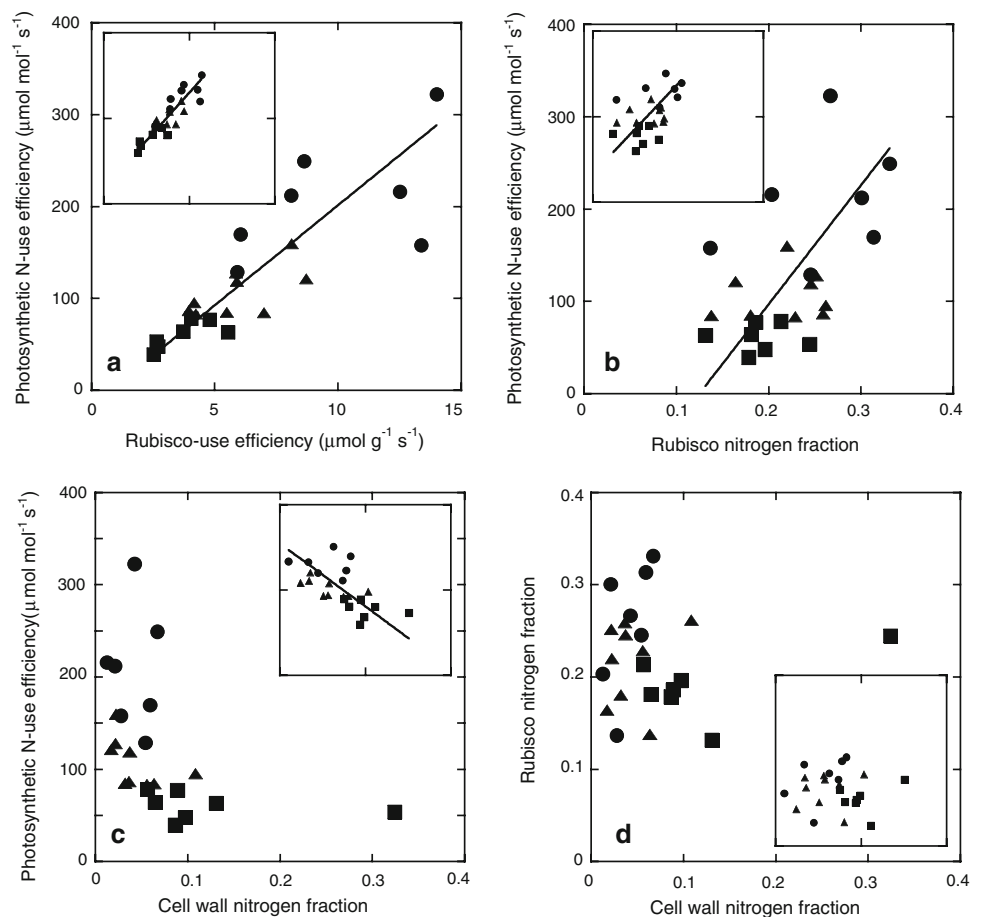
RBUE accounted for 70% of the variation in PNUE (Fig. 3). Subsequently, the variation in RBUE potentially involves Rubisco kinetics and  $\text{CO}_2$  diffusion to the carboxylation site. Stomatal conductance explained part of the variation; RBUE was positively correlated with the ratio of intercellular to air  $\text{CO}_2$  concentrations ( $C_i/C_a$ ) (Fig. 4a). The contribution of  $C_i$  to the variation in photosynthetic rate differs among previous studies. In some studies,  $C_i$  was similar among species irrespective of photosynthetic capacity (e.g., Yoshie 1986; Poorter and Farquhar 1994; Poorter and Evans 1998), whereas in other studies it was lower in

species with low PNUE (Hikosaka et al. 1998; Warren and Adams 2004b). In the Glopanet survey,  $C_i$  tended to be lower in species with lower photosynthetic capacity or higher LMA (Wright et al. 2004). These results suggest that stomatal conductance plays a significant role in the variation in PNUE, but its effect may be confounded by other factors such as habitat environment. Lower stomatal conductance in low PNUE species may contribute to higher water-use efficiency in photosynthesis (Field et al. 1983). However, the observed variation in RBUE was greater than that expected from the biochemical model of photosynthesis, where mesophyll conductance is assumed to be infinite (dotted line in Fig. 4a); the line fitted well for a few high-RBUE species, but not for others.  $C_i/C_a$  accounted for 43% of the range of RBUE, indicating that other mechanisms were also involved.

Mesophyll conductance has been suggested as being an important variable for the interspecific variation in photosynthesis (Lloyd et al. 1992; Hikosaka et al. 1998; Flexas et al. 2008; Warren and Adams 2006). A recent literature survey showed that the drawdown of  $\text{CO}_2$  concentration from intercellular spaces to the carboxylation sites is greater in sclerophytes than in non-sclerophytes, which may partly impose low PNUE in sclerophytes (Warren 2008). In Fig. 4b, we estimated the  $\text{CO}_2$  concentration at



**Fig. 3** Relationships between photosynthesis, Rubisco, and cell wall parameters. Photosynthetic nitrogen-use efficiency (PNUE) is plotted against **a** Rubisco-use efficiency (RBUE, photosynthetic rate per Rubisco), **b** Rubisco nitrogen fraction (RNF, leaf nitrogen allocated to Rubisco), and **c** cell wall nitrogen fraction (WNF, leaf nitrogen allocated to cell walls). **d** RNF plotted against WNF. Circles Herbs, triangles deciduous woody species, squares evergreen woody species. Insets Same variables both on  $\log_{10}$ -transformed scale, where a graduation means  $10^n$ . Regression lines were obtained with standardized major axis regression ( $Y = -16.3 + 21.8 X$ ,  $r^2 = 0.70$  and  $\log Y = 1.18 + 1.12 \log X$ ,  $r^2 = 0.78$  for PNUE–RBUE;  $Y = -161 + 1291 X$ ,  $r^2 = 0.27$  and  $\log Y = 3.44 + 2.11 \log X$ ,  $r^2 = 0.22$  for PNUE–RNF;  $\log Y = 1.07 + 0.737 \log X$ ,  $r^2 = 0.38$  for PNUE–RBUE)



**Fig. 4** **a** Rubisco-use efficiency (RBUE) as a function of the ratio of intercellular to atmospheric  $\text{CO}_2$  concentration ( $C_i/C_a$ ). Circles Herbs, triangles deciduous woody species, squares evergreen woody species. Dotted line Theoretical curve of the relationship between RBUE and  $C_i/C_a$ ; the equation is the Rubisco-limited photosynthesis model with Rubisco kinetic parameter values obtained from tobacco leaves assuming mesophyll conductance being infinite.  $C_a$  was assumed to be  $372 \mu\text{mol mol}^{-1}$ . Continuous line Standardized major axis regression ( $Y = -16.0 + 31.4 X$ ,  $r^2 = 0.61$ ). **b**  $\text{CO}_2$  concentration at the carboxyl-

ation site ( $C_c$ ) and the drawdown in  $\text{CO}_2$  concentration from intercellular spaces to the carboxylation sites ( $C_i - C_c$ ) as a function of RBUE. Dotted line Theoretical curve of the relationship between  $C_c$  and RBUE with assumptions that Rubisco kinetic parameter values followed actual values from tobacco leaves.  $C_i - C_c$  values were calculated as the difference between the measured  $C_i$  and the estimated  $C_c$ . Continuous line Normal regression of  $C_i - C_c$  on RBUE ( $Y = 189 - 8.04 X$ ,  $r^2 = 0.52$ )

the carboxylation site ( $C_c$ ) from RBUE, assuming that Rubisco was fully activated and values of Rubisco kinetics of the studied species were identical to those of tobacco (von Caemmerer et al. 1994).  $C_c$  increased with increasing RBUE (Fig. 4b), and the drawdown ( $C_i - C_c$ ) negatively correlated with RBUE. The calculated  $C_i - C_c$  values varied from 40 to 200  $\mu\text{mol mol}^{-1}$ , which were within the range of those shown by the literature survey (Warren 2008). These results suggest that mesophyll conductance contributed to the variation in RBUE. Interestingly, RBUE was negatively correlated with LMA and cell wall parameters (Table S1). This implies that thicker cell walls in mesophyll cells reduce mesophyll conductance for  $\text{CO}_2$  diffusion (Kogami et al. 2001; Terashima et al. 2006).

It should be noted, however, that the above calculation assumed Rubisco kinetics being identical among species. Although we did not study Rubisco kinetics, Hikosaka et al. (1998) reported that the specific activity of Rubisco was slightly but significantly different between an annual (*Chenopodium album*) and an evergreen tree (*Quercus myrsinaefolia*). Recently it was found that there is a significant interspecific variation in the Rubisco specificity factor, which was significantly correlated with the life form and the habitat xericity (Galmés et al. 2005). However, its variation was small and the dependence on the life form was weak; on average, the difference between annual herbs and evergreen trees was only 6% (Galmés et al. 2005). These facts suggest that the contribution of variation in Rubisco kinetics to the variation in RBUE may be small but significant, and the calculated  $C_c$  values of low-RBUE species in Fig. 4b may be underestimated.

Our data showed that nitrogen allocation plays a significant role in the variation in PNUE; high PNUE species invested more nitrogen in Rubisco (Fig. 3b). This is consistent with previous studies (Hikosaka et al. 1998; Poorter and Evans 1998; Westbeek et al. 1999; Pons and Westbeek 2004; Warren and Adams 2004b; Warren et al. 2006; Feng et al. 2007; Feng 2008). However, the correlation was weak compared with that expected from earlier studies. It should be noted that some previous studies have estimated Rubisco content from the relationship between photosynthetic rate and  $C_i$  (e.g., Pons and Westbeek 2004; Feng et al. 2007; Feng 2008; Niinemets and Tenhunen 1997), i.e., mesophyll conductance was implicitly assumed to be infinite. However, the drawdown from  $C_i$  to  $C_c$  is neither infinite nor constant across species as discussed above (Fig. 4b, Warren and Adams 2004a, 2006; Warren 2008). Such previous studies might have underestimated the Rubisco content especially for low-RBUE species.

Although cell wall nitrogen fraction (WNF) was negatively correlated with PNUE (Fig. 3c), there was no significant correlation between WNF and RNF (Fig. 3d). Studying many Australian tree species, Harrison et al.

(2009) also suggested that WNF did not affect nitrogen allocation to photosynthesis. These results contradict the hypothesis that there is a trade-off in nitrogen allocation between photosynthesis and cell walls (Hikosaka 2004). Increase in nitrogen allocation to cell walls will decrease PNUE if it reduces allocation to Rubisco (Onoda et al. 2004; Takashima et al. 2004), but this is not a general trend when we observe a broader range of species. Although nitrogen investment in cell walls is strongly related with LMA (Fig. 2d), it does not necessarily affect nitrogen allocation to photosynthesis. There may be another nitrogen sink in leaves other than the photosynthetic apparatus and cell walls. It is known that some species accumulate secondary nitrogenous compounds. For example, some *Eucalyptus* species invest nearly 20% of leaf nitrogen in cyanogenic glucosides (Gleadow et al. 1998). However, accumulation of secondary nitrogenous compounds is not necessarily general in other species. Such interspecific variations in nitrogen allocation to other functions may weaken the correlation between Rubisco and cell wall nitrogen.

Our results suggest that the cell wall is an important component in understanding the interspecific variation in leaf traits. Cell walls are an important nitrogen sink that varies in size along the variation in LMA (Fig. 2d), though it does not directly affect PNUE. Increasing cell wall contents may affect RBUE, which consequently causes a considerable decrease in photosynthetic capacity in species with high LMA (Fig. 1a,b). Greater cell wall contents may promote toughness of leaves (Reich et al. 1991; Wright and Cannon 2001; Onoda et al. 2008), tolerance to physical disturbance, and protection from herbivory and pathogens (Coley 1983), which in turn contributes to a longer leaf life span (Fig. 1f; Wright and Cannon 2001).

Our study demonstrated that the interspecific variation in PNUE is not caused by a sole factor that is markedly different among species. As a rough calculation, nitrogen allocation and stomatal conductance accounted for 27 and 30% of PNUE variation, respectively. The residual may be ascribed to mesophyll conductance and Rubisco kinetics. A previous study comparing PNUE between *Chenopodium album* (an annual) and *Quercus myrsinaefolia* (an evergreen tree) reached a similar conclusion; *Q. myrsinaefolia* had 14% lower  $C_i/C_a$ , 5–9% greater  $C_i - C_c$ , 23% smaller allocation of nitrogen to Rubisco, and 14% lower Rubisco specific activity, which resulted in a 50% decrease in PNUE compared with *C. album* (Hikosaka et al. 1998). Interspecific variation in PNUE thus results from small differences in several factors. Adaptations to stressful environments, such as high water-use efficiency, large allocation to secondary compounds, and tough leaves, may impose low PNUE. However, these adaptations enable longer leaf life span, which may contribute to the lifetime integral of nitrogen-use

efficiency due to longer nitrogen residence times (Hikosaka 2004, 2005).

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